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Short Communication

Differentiation of *Candida albicans* and *Candida dubliniensis* Using a Single-Enzyme PCR-RFLP Method

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SUMMARY: *Candida dubliniensis* is a novel *Candida* spp. that is similar to *Candida albicans* with respect to several phenotypic characteristics. However, they differ from each other with respect to epidemiology, pathogenesis, and the rapid development of resistance to fluconazole. In the present study, we used a single-enzyme PCR-restriction fragment length polymorphism (RFLP) technique to differentiate *C. dubliniensis* from *C. albicans*. The amplified ITS region of *C. dubliniensis* was digested once using the enzyme *Bln*I, whereas that of *C. albicans* remained intact. All standard strains tested were identified successfully by this method. None of 140 clinical isolates identified morphologically as *C. albicans* were recognized as *C. dubliniensis* based on their PCR-RFLP pattern. Our PCR-RFLP method easily differentiated *C. dubliniensis* from *C. albicans*, and this result was also demonstrated with standard strains.

Candida spp. are pathogenic yeasts in humans that continue to be of considerable medical importance. Over the last two decades, there has been a significant increase in the number of reported infections caused by yeasts of the genus *Candida*. These yeasts are associated with diseases ranging from superficial or mucocutaneous candidiasis to life-threatening systemic infections. *Candida albicans* is the most frequently isolated *Candida* spp. However, in recent years, the number of infections due to non-albicans *Candida* spp. has increased significantly.

Candida dubliniensis is a novel *Candida* spp. first described as a distinct taxon in 1995 (1). This species was associated primarily with oral candidiasis in patients infected with human immunodeficiency virus (HIV). However, unlike *C. albicans*, this species accounts for only approximately 2% of cases of candidemia, and is rarely isolated from the oral cavity microflora of healthy individuals (2).

Identification of *C. dubliniensis* still remains a problem in routine laboratories due to the high degree of phenotypic similarity between this species and *C. albicans*. Like *C. albicans*, *C. dubliniensis* can produce germ tubes and chlamydospores. These two species can be differentiated by examination of their phenotypic features, including a lack of growth at 45°C, intracellular beta-D-glucosidase activity, and carbohydrate assimilation profiling (2). However, these phenotypic tests are usually time-consuming and do not give completely reliable results. Hence, genotypic methods have been used for differentiating between these two species. For the molecular identification of *C. dubliniensis*, various methods have been described, including 25S ribosomal DNA analysis (3), PCR fingerprinting (4), molecular beacon assay (5), fluorescent probe hybridization (6), and amplified fragment length polymorphism analysis (7). With the exception of the study by McCullough et al., which introduced a simple molecular typing method for the differentiation of *C. dubliniensis* from *C. albicans* (8), most of the above-mentioned tests are labor-intensive or too expensive for routine use in medical laboratories.

According to the ambiguous epidemiological distribution of *C. dubliniensis* due to its close resemblance to *C. albicans*, as well as to the emergence of stable resistance to fluconazole in *C. dubliniensis* (2), a simple, reliable, and rapid method for discriminating between these two species is still required. Here, we report the application of a simple PCR-restriction fragment length polymorphism (RFLP) technique for differentiation between *C. albicans* and *C. dubliniensis*.

Standard *Candida* strains and the GenBank accession numbers of the sequences used in the present study are listed in Table 1. A total of 140 clinical isolates from the Medical Mycology Laboratory, Tehran University of Medical Sciences, identified as *C. albicans* by CHROMagar Candida, were also used. From the 140 clinical samples, 41% were isolated from male patients, and the rest were isolated from female patients. The average and median ages of the patients were 29.19 ± 11.14 and 27 years, respectively. The *C. albicans* isolates were from different body sites, including the skin and nails, mucosal membranes, and viscera, with frequencies of 70, 13, and 17%, respectively.

Genomic DNA was extracted and purified using glass bead disruption (9). Primers were selected to allow the amplification of the target ITS1-5.8s-ITS2 ribosomal DNA in both species. The primer sequences were: ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) for the forward primer, and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) for the reverse primer.

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Table 1. *Candida* standard strains and GenBank accession numbers used in this study

Standard strains	*Candida albicans*	ATCC 10261, ATCC 10231, ATCC 90029, ATCC 24432, ATCC 90028, TIMM 1768
	Candida dubliniensis	CBS 2747, CBS 7988, CBS 7987, CBS 8500, CBS 8501, IFM 49833, Ka3-1, Dm 42, H 38-2, Ka 12, Ka 6, H 39
GenBank sequences	*Candida albicans*	L47111, AB049122, AB049121, AB049120, AB049119, AF217609, AB018038, AB018037, AB032174, AB032173, AB032172, AJ249486
	Candida dubliniensis	AJ010332, AJ311898, AJ311897, AJ311896, AJ311895, AB049123, AB049124, AF430249, AJ249484, AB035590, AB035589, AJ249485

PCR amplification was carried out in a final volume of 50 μl. Each reaction contained 1 μl of template DNA, each primer at 0.5 μM, each deoxynucleoside triphosphate (dNTP) at 0.1 mM, 5 μl of 10 × PCR buffer, and 1.25 U of *Taq* polymerase. An initial denaturation step at 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min, with a final extension step of 72°C for 5 min. Amplified products were visualized by 1.2% (w/v) agarose gel electrophoresis in TBE buffer, and the products were stained with ethidium bromide.

A discriminatory restriction enzyme was selected according to an analysis of the recognition sites of various restriction enzymes within the region of interest. Briefly, the ITS sequences of 12 strains of *C. dubliniensis* and 12 *C. albicans* submitted to GenBank (Table 1) were aligned, and restriction patterns of the PCR products of both species were predicted for each of the known restriction enzymes using Genetyx software (version 6.1, Genetyx Corporation 2003, Tokyo, Japan). Finally, the enzyme *Bln*I (*Avr*II) (Roche Molecular, Mannheim, Germany) was selected to achieve the best species-specific fragment length pattern.

Digestion was performed by the incubation of 21.5- μl aliquots of PCR products with 10 U of the enzymes in a final reaction volume of 25 μl at 37°C for 2.5 h. Restriction fragments were separated by 2% agarose gel electrophoresis in TBE buffer for approximately 1 h at 100 V, and the samples were stained with ethidium bromide.

The primers successfully amplified the ITS region of all yeasts tested, providing a single PCR product of the expected size (540 bp). There were no visible differences between these two species with regard to their ITS PCR product (Fig. 1). The PCR amplicons were digested with *Bln*I as described in Materials and Methods. The products of digestion are shown separately in Fig. 2, indicating that the bands generated corresponded to the predicted sizes, which were 540 bp for *C. albicans*, and 200 and 340 bp for *C. dubliniensis*. That is, *Bln*I has one recognition site within the ITS region of *C. dubliniensis*, whereas it has no digestion site within that of *C. albicans*.

Using this restriction enzyme, it was possible to distinguish between these two species. All *C. albicans* and *C. dubliniensis* standard strains had a similar pattern, and there was no variation among standard strains. None of the 140 clinical isolates identified morphologically as *C. albicans* were recognized as *C. dubliniensis* based on their PCR-RFLP pattern.

Conventional phenotypic methods based on the reported morphological and physiological characteristics have been

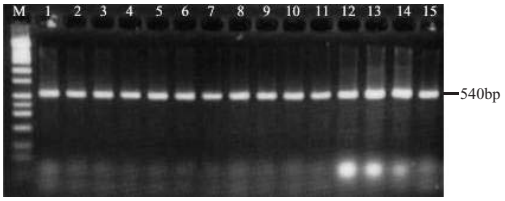

Fig. 1. Ethidium bromide-stained gel image of the ITS region. M, 100 bp ladder molecular size marker; lanes 1-11, *C. dubliniensis*; lanes 12-15, *C. albicans*.

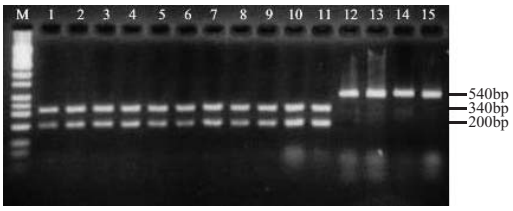

Fig. 2. PCR-RFLP patterns after digestion of PCR products with *Bln*I. M, 100 bp ladder molecular size marker; lanes 1-11, *C. dubliniensis*; lanes 12-15, *C. albicans*.

widely applied to the genus *Candida* (10). However, such methods are usually time-consuming and lack sufficient discriminatory power (7,11). Therefore, various molecular methods for characterizing species of *Candida* have been used (2-7). On the basis of the amplified 25S rDNA length, *C. albicans* was classified into four groups (A-D) (8). Using this simple method, a new genotype (E), which is thought to have been derived from a possible horizontal transfer of the group I intron between *C. albicans* and *C. dubliniensis*, was identified within the *C. albicans* strains (12).

In the present study, we used the ITS region, which contains several regions of relatively well-conserved sequences useful to obtain proper sequence alignments, and yet the ITS region also contains sufficient sequence variability such that the non-homologous sequences can serve as markers for species-specific RFLP. This region has been used completely or partially by other investigators for the species identification of some medically important fungi. Previously, we reported a single-enzyme PCR-RFLP method using ITS1 and ITS4 primers for the discrimination of six well-known pathogenic *Candida* spp. (13). Here, we used an additional enzyme for the differentiation of *C. dubliniensis*, which was formerly classified as *C. albicans*. Although the molecular typing method reported by Tamura et al. (12) is simpler, the universal primers used in the present study are more general and are also available in almost all molecular mycology

laboratories. Using this universal PCR and an appropriate restriction enzyme, the expansion of this identification system is feasible.

In the present study, we analyzed and identified standard strains of yeast by digestion of the ITS region with *Bln*I; no band variation was seen within the standard strains of the same species examined. Moreover, the results of PCR-RFLP analysis of the digested ITS sequences of 24 *Candida* strains (12 strains of *C. dubliniensis*, 12 strains of *C. albicans*) obtained from GenBank-NCBI were comparable to those from the standard isolates. We recommend this profile as a simple and rapid method for differentiation between two morphologically similar species, *C. albicans* and *C. dubliniensis*.

C. dubliniensis isolates have been primarily recovered from oral and mucosal surfaces, especially in HIV-positive patients (1,2,10). However, there have been a number of recent reports of its isolation from non-HIV-positive patients (5,14). In the present study, we failed to isolate *C. dubliniensis* from clinical samples. The differences in the results of the present study and those of previous studies may also reflect differences in geographical locations and patient populations. There were no HIV-positive cases in our study sample, and a small number of isolates were from the oral cavity or other mucosal membranes. Therefore, further studies will still be needed in order to determine the prevalence of *C. dubliniensis* in Iran.

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